Discrimination of tilapia species of the genera *Oreochromis*, *Tilapia* and *Sarotherodon* by PCR-RFLP of 5S rDNA

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5S rDNA sequences present an intense dynamism in the genome and have proved to be valuable as genetic markers to distinguish closely related taxa. In order to identify patterns of 5S rDNA variation useful in the discrimination of tilapia species of the genera *Oreochromis, Tilapia* and *Sarotherodon* we applied PCR-RFLP of 5S rDNA repeat units in the tilapiine species *O. niloticus* (Linnaeus), *O. karongae* (Trewavas), *O. aureus* (Steindachner), *O. mortimeri* (Trewavas), *O. mossambicus* (Peters), *S. galilaeus* (Linnaeus), *T. rendalli* (Boulenger) and *T. zillii* (Gervais). The PCR-RFLP results obtained validate the use of 5S rDNA polymorphisms to discriminate tilapia species and genera.

The group of cichlid fish known as tilapias have been used for a long time as an important source of animal protein by humans. Although tilapias comprise over 70 species (Trevawas, 1983), *Oreochromis niloticus, O. mossambicus, O. aureus* and *Tilapia rendalli* are the most important species employed in farming, along with certain interspecies hybrids. The systematics of the group is based on morphological, ecological and behavioral parameters and species identification in this group can be very difficult. Several tilapiine species share similar morphological features and can be easily hybridized (Greenwood, 1991; Galls & Metz, 1998), and considerable interpopulation variation has been detected in many species (Bardakci & Skibinski, 1994). These factors have caused several problems in identification, especially concerning species of aquaculture importance or cases of hybridization. For these reasons, genetic markers that can identify and discriminate species in this group are of high potential value to fundamental and applied studies of these fishes.

Allozyme variation has been used for tilapia species identification (McAndrew & Majumdar, 1983), in phylogenetic studies (e.g. Sodsuk & McAndrew, 1991; Pouyaud & Agnese, 1995) and studies on hybridization and introgression between species (e.g.

Mather & Arthington, 1991; Gregg, Howard & Shonhiwa, 1998; Adepo-Gourene, Gourene & Agnese, 2006) but these markers present considerable difficulties for collection and storage (fish need to be killed in most cases; tissues need to be kept frozen at low temperatures until analysed) in comparison to DNA markers amplified by PCR (small biopsies are generally sufficient; these can be stored in ethanol without freezing).

Sequencing of specific regions of mitochondrial DNA (mtDNA) can be used to discriminate between tilapia species and thus for both phylogenetic studies (e.g. Nagl, Tichy, Mayer, Samonte, McAndrew & Klein, 2001; Klett & Meyer, 2002) and to analyse hybridization and introgression (Rognon & Guyomard, 2003; D'Amato, Esterhuyse, van der Waal, Brink & Volckaert, 2007), but the application of mtDNA in studying hybridization and introgression is limited due to its maternal inheritance. Microsatellite DNA markers, although nuclear and thus showing biparental inheritance, generally exhibit large numbers of alleles which limit their usefulness in clearly discriminating tilapia species and in studying hybridization (D'Amato et al., 2007). There are few publications on other types of nuclear DNA markers that can distinguish between species of tilapias (e.g. RAPD: Bardakci & Skibinski, 1999; Ahmed, Ali & El-Zaeem, 2004).

Ribosomal 5S rDNA has already proved its applicability in species identification, for example in fish of commercial and ecological interest such as salmon, trout (Pendas, Móran, Martínez & Garcia-Vásquez, 1995; Carrera, Garcia, Céspedes, González, Fernández, Asensio, Hernández & Martin, 2000) and sharks (Pinhal, Gadig, Wasko, Oliveira, Ron, Foresti & Martins, 2008). The value of 5S rDNA sequences as markers comes from the combination of its genome organization and its characteristic evolutionary pattern. In higher eukaryotes, the 5S ribosomal DNA consists of multiple

copies of a highly conserved 120 base pairs (bp) coding sequence, separated from each other by a variable non-transcribed spacer (NTS) (Long & David, 1980). The copies are organized in a head-to-tail direction and the NTSs are flanked by the 5S rRNA gene, thus the NTS can be easily amplified by PCR. Another characteristic is that the 5S rRNA gene is highly conserved, even among non-related taxa, which make it possible to isolate the 5S rRNA genes of one species based on the available sequence of another non-related one, simply by the use of PCR. Therefore, we focus in this paper on the development of a simple and reliable technique, based on 5S rDNA variation, to discriminate tilapia species of the genera *Oreochromis*, *Tilapia* and *Sarotherodon* .

All specimens analyzed were obtained from the Institute of Aquaculture, University of Stirling: five *O. niloticus* (two XX females, two XY males, and one YY "supermale"); two *O. karongae* (one male and one female); one *O. aureus* (male); two *O. mortimeri* (females); two *O. mossambicus* (females); two *T. rendalli* (one male and one female); two *T. zillii* (one male and one female)*;* and one *S. galilaeus* (male).

Genomic DNA was extracted from livers according to Sambrook and Russell (2001). PCR amplifications of repeat units of 5S rDNA were performed as described by Alves-Costa, Wasko, Oliveira, Foresti & Martins (2006), using primers, 5SA (5'-TAC GCC CGA TCT CGT CCG ATC - 3') and 5SB (5' - CAG GCT GGT ATG GCC GTA AGC-3') designed from the rainbow trout 5S rRNA sequence (Komiya & Takemura, 1979).

The amplified samples (and a negative control) were checked in a 1% agarose gel (1xTAE buffer; TRIS-acetate 0.04 M /1 mM EDTA, pH 8.3). PCR amplification generated ~500 bp amplicons for all the analyzed samples. They were then analysed by Restriction Fragment Length Polymorphism (RFLP) using 14 restriction enzymes (*Apa*I, *Knp*I, *Hae*III, *Bc*II, *Eco*RV, *Msp*I, *Acc*65I, *Hind*III, *Eco*RI, *Ps*tI, *Pvu*II, *Hinf*III,

*Sac*I. The DNA fragments were analyzed through agarose and polyacrylamide gel electrophoresis.

Out of the 13 examined enzymes, eight did not cleave the 5S rDNA-PCR products (*Apa*I, *Knp*I, *Hae*III, *Bc*II, *Eco*RV, *Msp*I, *Acc*65I, *Sac*I). Two did cleave amplicons (*Hind*III and *Eco*RI), but the three fragments generated were of equal sizes for all samples (an upper band corresponding to undigested products, a fragment of ~400 bp and of ~100 bp).

Three enzymes (*Pvu*II, *Pst*I, *Hinf*III) gave polymorphic patterns that were useful as markers to distinguish the tilapia genera and species investigated (Fig. 1). All of the *Oreochromis* species had a similar band pattern after digestion by *PstI*, *PvuI* and *Hinf*III, with small differences in the DNA fragments generated. All *Oreochromis* specimens had a ~500 bp band corresponding to undigested amplicons for the enzymes *Pvu*II and *Pst*I. There could also be noted one band sized about ~330-350 bp and one ~120-140 bp generated by the enzyme *Pvu*II. The ~330-350 bp band pattern, was able to discriminate *O. niloticus* from the other *Oreochromis* species (*O. karongae*, *O. aureus*, *O. mortimeri* and *O. mossambicus*), due to a small (~20 bp) difference in size. On *Pst*I digestion, *O. niloticus* also showed small differences in relation to the other *Oreochromis* species. The band pattern originated by *PstI* has the size of ~370 and ~380 bp for *O. niloticus* and the other *Oreochromis* species respectively. The enzyme *Hinf*III gave only one band of ~480 bp band for *O. niloticus* and a band of ~500 bp for the other *Oreochromis* species (Fig. 1). For both *PstI* and *PvuI* (and also *Hind*III and *Eco*RI, as described above) the presence of an undigested ~500 bp band is related to the presence of two classes of 5S rDNA in the tilapia species (Martins, Wasko, Oliveira & Wright, 2000; Martins, Wasko, Oliveira, Porto-Foresti, Parise-Maltempi, Wright & Foresti,

2002; Alves-Costa et al., 2006), one of them being digested and generating the band pattern observed.

The enzymes *Pvu*II, *Pst*I, *Hinf*III also distinguished the genera *Tilapia* and *Sarotherodon* from *Oreochromis* (Fig. 1). All of the species examined of these two genera lacked a cleavage site for *Pvu*II, reflected in the single undigested band observed. *T. zillii* was clearly discriminated by the presence of two bands (~300 and 200 bp), plus the ~500 bp one containing non-fragmented amplicons, following digestion by *Pst*I. These bands represent a clear pattern that can be applied as a marker of *T. zillii* against any of other tilapia species analysed.

Digestion with *Hinf*III also give informative results on the discrimination of tilapiini species (Fig. 1). For the genera *Oreochromis* and *Sarotherodon*, a single bright band of ~480 bp was observed. This band was absent in *Tilapia*, allowing the discrimination of *Tilapia* from the other two tilapiini genera. On the other hand, *Tilapia* and *Sarotherodon* presented two bands of ~300 and ~100 bp. The band pattern produced by *Hinf*III allowed the discrimination of the three tilapia genera. Table 1 summarizes the results obtained, and shows that of 28 species pair comparisons, the PCR-RFLP patterns shown in Figure 1 were capable of discrimination in 22 cases.

A good explanation for the variation in restriction patterns observed for the 5S rDNA repeats is the presence of point mutations or even small insertions/deletions that are common in the NTS of 5S rDNA sequences (Martins & Wasko, 2004). NTSs in general are considered to show great nucleotide variability attributed to insertions/deletions, minirepeats, and pseudogenes (Nelson & Honda, 1985; Leah, Frederiksen, Engberg & Sorensen, 1990; Sajdak, Reed & Phillips, 1998).

The present data demonstrates the potential applicability of the 5S rDNA sequences as a genetic marker for the differentiation of several tilapia species across

three genera. It is also worth mentioning that particularly in fishery management and conservation, the 5S rDNA PCR-RFLP approach is relatively easy to apply. Additionally, such technology could be applied on fish products that are commonly sold in markets, allowing an approach to the identification of species.

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Figure Legend

Figure 1. 5S rDNA PCR-RFLP profiles of YY *O. niloticus* (1), XX *O. niloticus* (2, 3), XY *O. niloticus* (4, 5), *O. aureus* (6), *O. karongae* female (7), *O. karongae* male (8), *O. mortimeri* male (9), *O. mortimeri* male (10), *O. mossambicus* male (11, 12), *T. zillii* female (13), *T. zillii* male (14), *T. rendalli* male (15), *T. rendalli* female (16), *S. galilaeus* male (17). Restriction digestion with *Pvu*II (a), *Pst*I (b) and *Hinf*III (c). Molecular weight markers in bp are shown on the left (M).

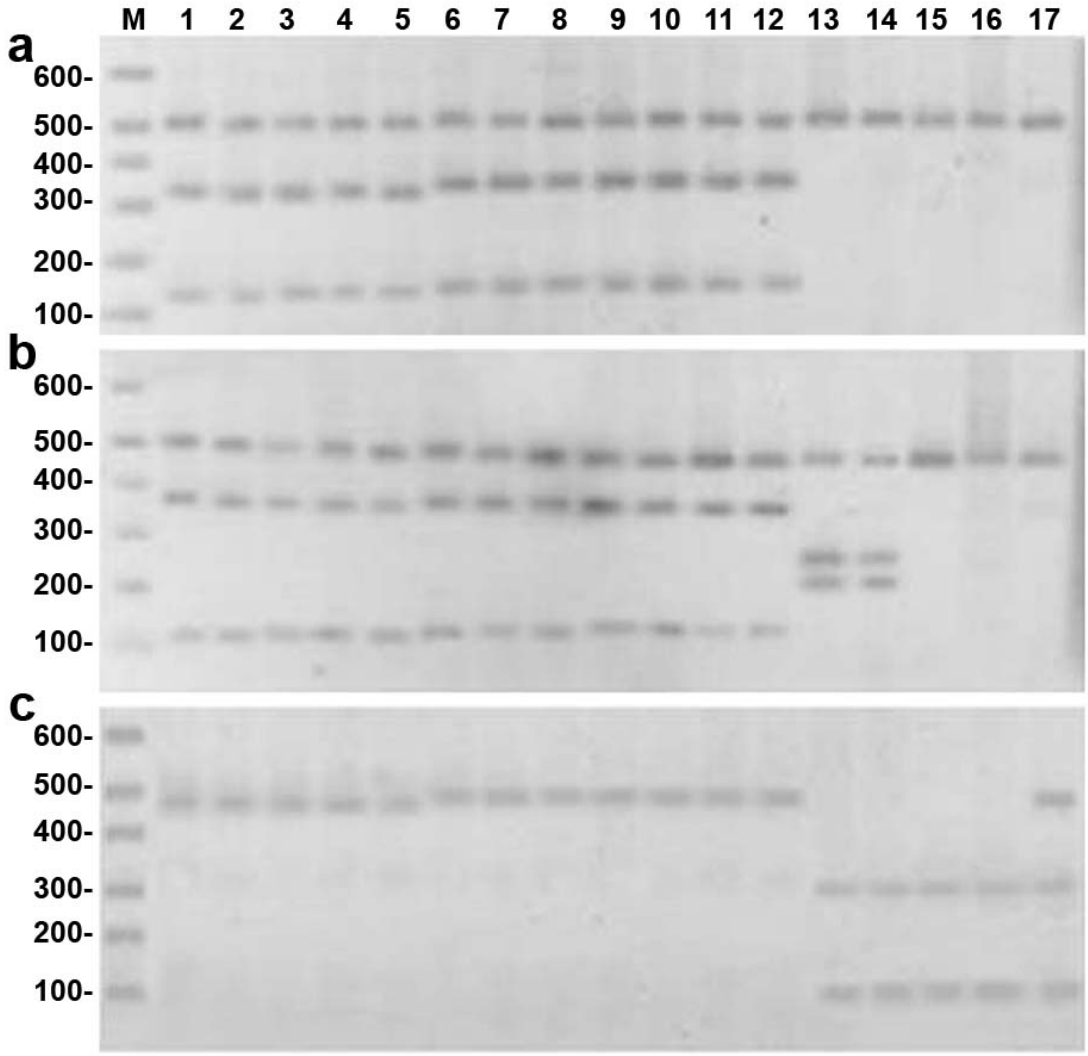

Table 1. Summary of discrimination between tilapia species using PCR-RFLP patterns of 5S rDNA repeat units of *O. niloticus* (nil), *O. aureus* (aur); *O. karongae* (kar), *O. mortimeri* (mort), *O. mossambicus* (moss), *T. rendalli* (rend), *T. zillii* (zill) and *S. galilaeus* (gal). The discrimination between species with the enzymes *Pvu*II, *Pst*I and *Hinf*III is indicated with the letter a, b and c respectively. X represents the absence of discrimination between species pairs using any of the three enzymes.

	nil	**aur**	**kar**	**mort**	**moss**	**zill**	**rend**	**gal**
nil	-	abc	abc	abc	abc	abc	abc	abc
aur		-	X	X	X	abc	abc	abc
kar			-	X	X	abc	abc	abc
mort				-	X	abc	abc	abc
moss					-	abc	abc	abc
zill						-	b	bc
rend							-	c
gal								-